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A Hox-Eya-Pax Complex Regulates Early Kidney Developmental Gene Expression▽†

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During embryonic development, the anterior-posterior body axis is specified in part by the combinatorial activities of *Hox* genes. Given the poor DNA binding specificity of Hox proteins, their interaction with cofactors to regulate target genes is critical. However, few regulatory partners or downstream target genes have been identified. Herein, we demonstrate that Hox11 paralogous proteins form a complex with Pax2 and Eya1 to directly activate expression of *Six2* and *Gdnf* in the metanephric mesenchyme. We have identified the binding site within the *Six2* enhancer necessary for Hox11-Eya1-Pax2-mediated activation and demonstrate that this site is essential for *Six2* expression in vivo. Furthermore, genetic interactions between *Hox11* and *Eya1* are consistent with their participation in the same pathway. Thus, anterior-posterior-patterning Hox proteins interact with Pax2 and Eya1, factors important for nephrogenic mesoderm specification, to directly regulate the activation of downstream target genes during early kidney development.

The *Hox* genes are conserved among all metazoans and specify positional information along the body axes. In mammals, 39 *Hox* genes are arranged into four chromosomal clusters, which are organized into 13 paralogous groups along the chromosome in a 3′-to-5′ manner. This arrangement of genes leads to spatiotemporal colinearity of *Hox* gene expression, with 3′ genes expressed more anteriorly and earlier in development than 5′ genes (16, 28). The unique, combinatorial expression of Hox proteins is important for the regulation of anterior-posterior patterning, skeletal morphogenesis, mesodermal organ development, neural patterning, and multiple other cellular and developmental processes (reviewed in reference 64). Despite genetic analyses of a variety of organisms, the molecular details regarding which cofactors interact with Hox proteins to regulate transcription are poorly understood, and very few downstream Hox targets have been identified.

The mammalian kidney serves as an ideal model organ to study Hox protein function. The embryonic kidney is derived from the intermediate mesoderm and exhibits anterior-posterior patterning as it develops (reviewed in reference 14). The most anterior region of the intermediate mesoderm, the pronephros, is a rudimentary structure in mammals. Caudal to the pronephros are the mesonephric tubules, a linear array of nephron-like structures that are transient filtering units. The most posterior intermediate mesoderm generates the metanephric, or adult, kidney, which forms adjacent to the hindlimb buds. Adult kidney development begins when the metanephric mesenchyme induces an outgrowth, the ureteric bud, from the adjacent nephric duct epithelia. The ureteric bud invades the metanephric mesenchyme, provides inductive Wnt signals, and subsequently undergoes branching morphogenesis to generate the radial pattern of the kidney (reviewed in reference 75). In mice, *Hox11* paralogous genes are essential for early patterning of the metanephric mesenchyme, as the loss of *Hoxa11* and *Hoxd11* results in misrouted ureters and hypoplastic kidneys, while the loss of all three *Hox11* paralogs results in a complete failure of the metanephric mesenchyme to induce ureteric bud induction (11, 46, 70).

Hox proteins recognize a degenerate ATTA or TTAT sequence, which confers very little locus specificity (17; reviewed in reference 64). Thus, it is likely that cofactors interact with Hox proteins in order to promote high-affinity binding and locus-specific regulatory activities. The three-amino-acid-loop extension proteins Pbx and Meis/Prep are known Hox cofactors that regulate target gene expression (reviewed in reference 39). *Pbx1*, *Pbx3*, and *Meis1* are expressed in the kidney; however, the loss of *Pbx1* and *Meis* function results in kidney phenotypes that are less severe and affect later stages than those of the *Hox11* paralogous gene mutants (12, 24, 60, 61). Thus, it is likely that Hox11 proteins are interacting with other cofactors to specify the early intermediate mesoderm along the anterior-posterior axis.

Prior to ureteric bud induction, the condensing metanephric mesenchyme expresses a unique combination of markers, including the *Hox11* paralogs (*Hoxa11*, *Hoxc11*, and *Hoxd11*), *Osr1*, *Pax2*, *Eya1*, *Wt1*, *Six1*, *Six2*, and *Gdnf* (14). In *Hox11* triple mutants, the expression of many early kidney patterning markers in the uninduced metanephric mesenchyme are unperturbed; however, both *Six2* expression and *Gdnf* expression are absent (70). *Six2* regulates metanephric progenitor cell renewal (63). Gdnf ligand activates coreceptors c-ret and Gdnfrα in the Wolffian duct to promote ureteric bud outgrowth and invasion and is a key regulator of continued branching morphogenesis (6, 23, 40, 50, 58, 59, 62, 66, 67). *Pax2* mutant mice have reduced levels of *Six2* expression and do not

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express *Gdnf* in the mesenchyme (65). Even though Pax2 can directly regulate *Gdnf* expression in cell culture, it is not sufficient in vivo, as *Gdnf* expression is absent in *Hox11* and *Eya1* mutants, while *Pax2* expression is normal in these mutants (5, 70, 71). *Eya1* mutants, like *Hox11* mutants, exhibit no ureteric bud induction and also lack *Six2* and *Gdnf* expression (71).

The *Pax*, *Eya*, and *Six* gene families encompass members of a common regulatory network that is conserved from *Drosophila melanogaster* to mammals (reviewed in reference 3). Eya proteins have no intrinsic DNA binding domain but can localize to the nucleus and function as coactivators of transcription (43, 51). The Eya1 protein also has a protein phosphatase activity which is essential for the regulation of some target genes (reviewed in reference 55).

Given the similarities in molecular phenotypes observed in the *Hox11*, *Pax2*, and *Eya1* mouse mutants in the developing kidney, we propose that Hox11 proteins interact with Pax2 and Eya1 to regulate essential patterning genes within the posterior intermediate mesoderm. All three proteins physically interact and synergistically up-regulate *Gdnf* and *Six2* promoter activities. We identified a binding site critical for this activation within the *Six2* promoter region and demonstrated that this site is essential for driving expression within the renal mesenchyme. Furthermore, renal development is sensitized to a partial loss of *Hox11* paralogous gene function in an *Eya1* heterozygous genetic background, suggesting that these proteins work in the same regulatory network. These data point to a novel Hox11-Eya1-Pax2 network that translates anterior-posterior positional information within the developing mammalian kidney. Further, our data demonstrate that *Six2* and *Gdnf* are direct downstream targets of the group of *Hox11* paralogous genes.

MATERIALS AND METHODS

Six2- and Gdnf-luciferase constructs. The 3.03-kb Six2 promoter region, at base pairs −266 to −3296 upstream of the ATG start site, was PCR amplified from a *Six2* bacterial artificial chromosome clone and inserted into a pGL3-Basic vector (Promega). A previously reported *Gdnf* promoter (5) was subcloned into pGL3-Basic. The QuikChange II XL site-directed mutagenesis kit (Stratagene) was used to mutate the *Pax2* binding site or delete the *Hox* binding site in the 3.03-kb Six2 promoter-luciferase vector. Mutations and deletions were confirmed by sequence analysis (see materials and methods in the supplemental material).

Protein expression vectors. The Hoxa11 protein coding sequence (NCBI IMAGE clone 8734051) was cloned into a p3XFlag-CMV10 expression vector (Sigma), which places three FLAG tags at the N terminus of Hoxa11. The Eya1 protein coding sequence (NCBI IMAGE clone 6848408) was cloned into a pCS2+MT expression vector, which places six Myc tags at the N terminus of Eya1 (57, 68). The Pax2 protein expression vector was previously described (34).

Luciferase assays. MDCK cells were plated at 50,000 cells per well in a 24-well plate, cultured in minimum essential medium with 10% fetal bovine serum, 50 IU of penicillin per ml, and 50 μg of streptomycin per ml at 37°C, and transfected the second day with FuGENE6 (Roche). For each well, 0.64 ml of FuGENE was used per 0.32 mg of DNA, which contained 75 ng of reporter plasmid and 20 ng pRL-CMV renilla luciferase for standardization. Twenty-four hours posttransfection, cells were lysed in Passive lysis buffer (Promega). Luciferase activity was measured using a Vector3 PerkinElmer luminometer. Counts were standardized using pGL3 empty vector or cotransfected renilla luciferase (Dual-Luciferase assay system; Promega). Each transfection was performed in triplicate.

Northern blot analysis. RNA from MDCK cells was prepared using TRIzol (Invitrogen) and resuspended in RNA storage solution (Ambion). Twenty micrograms of RNA per sample was electrophoresed for 2 h in 1% agarose denature gel (Ambion), blotted onto a Hybond-N+ membrane (Amersham Biosciences), and prehybridized with 8 ml ULTRAhyb ultrasensitive hybridization buffer (Ambion). The membrane was then hybridized at 68°C overnight with 8×10^6 cpm of a *Six2* RNA probe (HindIII-SfoI fragment from NCBI IMAGE clone 6394139 [ATG to bp 346 of exon 1]) labeled with [^{32}P]dUTP via in vitro RNA probe reverse transcription. The blot was washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.1% sodium dodecyl sulfate at 37°C and then twice with 0.1× SSC plus 0.1% sodium dodecyl sulfate at 68°C and exposed to film or analyzed on a Typhoon 9400 variable-mode imager (Amersham). The RNA probes for the *Hox11* paralogs, *Eya1*, and *Pax2* have been previously published (15, 26–28, 72).

Coimmunoprecipitation assays. HEK-293 cells were transfected with Hoxa11-FLAG, Pax2-hemagglutinin (HA), and Eya1-Myc expression vectors, and cell lysates were prepared (Complete Mini; Roche). Mouse anti-HA monoclonal antibody (catalog no. H9658; Sigma), mouse anti-FLAG monoclonal antibody (M2) (catalog no. F3165; Sigma), or mouse anti-Myc monoclonal antibody (catalog no. SC-40; Santa Cruz) was added to 0.5 μg lysate and incubated 4°C overnight. Mouse or rabbit immunoglobulin G (Jackson ImmunoResearch) was used as a negative control. Protein was precipitated using protein G agarose beads (Invitrogen), washed, and resuspended. Western blots were probed with 1:5,000 anti-FLAG antibody for Hoxa11, 1:3,000 anti-Pax2 antibody (catalog no. PRB-276P; Covance) for Pax2, or 1:500 anti-Myc antibody for Eya1.

Pax2-PD pull-down and electromobility shift assays. The 3.03-kb *Six2* upstream region was digested by HpaII and used in the Pax2-paired domain (Pax2-PD) pull-down assay following methods described previously (4).

Gel electromobility shift assays were performed as previously described with modifications (5). Nuclear extracts from untransfected HEK-293 cells and cells transfected with the Hoxa11-FLAG, Pax2-HA, and Eya1-Myc protein expression vectors were isolated as previously described (49). Polyacrylamide gel electrophoresis-purified oligonucleotides (Invitrogen) for the wild-type and mutant probes (see materials and methods in the supplemental material) were annealed and end labeled with P-32 (T4 polynucleotide kinase; Promega). Supershifts were performed using anti-Hoxa11 antibody (7) and anti-HA antibody (see above).

Six2-LacZ transgenic mice. The 3.03-kb wild-type and mutant upstream *Six2* sequences from the luciferase vectors were subcloned upstream from the β-galactosidase gene in the pNASSβ expression vectors (BDB Clontech) and were digested with NheI and KpnI to create 980-bp Six2-LacZ wild-type and mutant pNASSβ reporter vectors. DNA for injection was isolated using NheI and AseI. Purified DNA was microinjected into fertilized eggs obtained by mating $(C57BL/6 \times SJL)F_1$ or C57BL/6 female mice with $(C57BL/6 \times SJL)F_1$ male mice. Pronuclear microinjection was performed as described previously (41). Embryos were collected at embryonic day 11.5 (E11.5), genotyped for the β-galactosidase gene (2), and stained for β-galactosidase activity by following standard protocols (41).

In situ hybridization and histology. In situ hybridization and histology were performed as previously described (70).

RESULTS

As *Six2* expression is affected in *Hox11*, *Eya1*, and *Pax2* mutant metanephric mesenchymes, we tested whether the proteins encoded by these genes can activate *Six2* expression directly. A 3-kb sequence upstream of the *Six2* ATG start site was used to determine whether *Six2* expression is regulated in cell culture by Hox11, Pax2, or Eya1 (Fig. 1A). In transfected cells, Hoxa11, Eya1, or Pax2 alone was unable to substantially increase expression from the *Six2* reporter construct. However, when the proteins were coexpressed in combination, activation of the reporter was observed. Coexpression of all three proteins resulted in 50-fold activation of the *Six2* luciferase reporter (Fig. 1B). Expression of Hoxc11 and Hoxd11 vectors in place of Hoxa11 in these experiments showed similar results, consistent with their redundant genetic function at this early stage of kidney development (see Fig. S1 in the supplemental material) (70). In MDCK cells transiently transfected with Hoxa11, Pax2, and Eya1, a fivefold up-regulation of endogenous *Six2* expression was also demonstrated (Fig. 1C). Untransfected MDCK cells had measurable levels of endogenous *Pax2* mRNA expression but low to undetectable levels of *Hoxa11*, *Hoxc11*, *Hoxd11*, and *Eya1* expression (see Fig. S2 in the supplemental material).

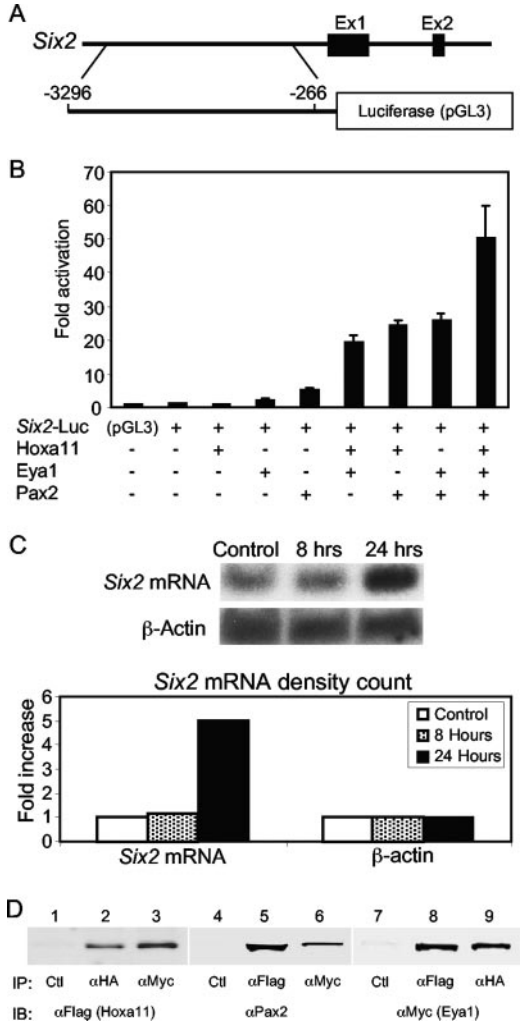

FIG. 1. Regulation of Six2 expression by a Hox11-Eya1-Pax2 complex. (A) Schematic of the Six2-luciferase vector. A fragment from base pair 3296 to base pair 266 upstream of the *Six2* ATG start site was subcloned into a luciferase expression vector. Ex1, exon 1; Ex2, exon 2. (B) Activity of the *Six2* luciferase reporter plasmid in transfected MDCK cells with different combinations of Hoxa11, Pax2, and Eya1 protein expression vectors. (C) Northern blot analysis of endogenous *Six2* mRNA in MDCK cells after transfection with Hoxa11, Eya1, and Pax2, normalized to β-actin. (D) Whole-cell extracts of HEK-293 cells transfected with Hoxa11-Flag, Pax2-HA, and Eya1-Myc protein expression constructs were subjected to reciprocal coimmunoprecipitations. Immunoblotting (IB) for Hoxa11 (αFlag) demonstrated coimmunoprecipitation with Pax2 (αHA) and Eya1 (αMyc) (lanes 2 and 3). Immunoblotting with Pax2 (αPax2) demonstrated coimmunoprecipitation of Hoxa11 (αFlag) and Eya1 (αMyc) (lanes 5 and 6). Immunoblotting with Eya1 (αMyc) demonstrates coimmunoprecipitation with Hoxa11 (αFlag) and Pax2 (αHA) (lanes 8 and 9). Immunopreciptations (IP) using mouse or rabbit immunoglobulin G (lanes 1, 4, and 7) were negative controls (Ctl).

The cooperative activation of *Six2* could be mediated by the formation of a Hox11-Eya1-Pax2 complex binding directly to an upstream *cis* regulatory sequence. To test for physical interactions, we performed coimmunoprecipitation experiments using Hoxa11, Pax2, and Eya1. In cell lysates expressing Hoxa11, Eya1, and Pax2, immunoprecipitation using antibodies specific for one of the three proteins resulted in the coprecipitation of the other two proteins (Fig. 1D). Thus, Hoxa11, Pax2, and Eya1 can form a complex and physically associate either directly or through interactions with as yet unidentified adaptor proteins within the complex.

To examine the possibility that the Hox11-Eya1-Pax2 complex could bind directly to sequences upstream of the *Six2* coding sequence, we first tested for Pax2 binding, since Pax2 is expected to have the most specificity in terms of a DNA recognition sequence, and the Pax2-PD has been previously shown to bind Pax2 target sequences with high affinity (5, 34). After digestion of the 3.0-kb *Six2* reporter sequence with HpaII, two fragments showed strong binding by the Pax2-PD in vitro: one at the 5′ end of the reporter construct and another sequence 450 base pairs upstream of the *Six2* coding sequence (Fig. 2A). Subsequent deletion analyses showed that the 5′ putative binding site was not required for Hox11-Eya1-Pax2-mediated activity in cell culture (data not shown). Sequence analysis of the bp −450 region binding site revealed a conserved *Pax2* consensus sequence (5, 18, 38, 48, 49) and an adjacent putative *Hox* binding site (Fig. 2B).

We next tested the necessity of these binding sites for reporter gene activation in our reporter assay. In addition to testing the wild-type 3-kb *Six2* luciferase constructs tested previously, we generated and tested three analogous 3-kb constructs with a mutation of the putative *Pax2* binding site at the bp −450 region, a deletion of a nearby putative *Hox* binding site, or both mutations together. Mutation of the *Pax2* or the *Hox* binding site alone caused a decrease in Hox11-Eya1-Pax2-mediated luciferase activity compared to the activity of the wild-type *Six2* construct. However, Hox11-Eya1-Pax2-mediated expression from the *Six2* reporter was nearly abolished when both the putative *Pax2* and *Hox* sites were mutated (Fig. 3A).

Binding at the bp −450 region site by the Hox11-Eya1-Pax2 complex was further examined by using nuclear extracts from HEK-293 cells transfected with Hoxa11, Eya1, and Pax2 in electrophoretic mobility shift assays (Fig. 3B). An 89-base-pair fragment containing the putative binding site exhibited a slower-migrating complex upon incubation with nuclear lysate expressing Hoxa11, Eya1, and Pax2 (Fig. 3B, lane 3). This slower-migrating species was supershifted upon incubation with antibodies against Hoxa11 or Pax2, indicating that these proteins are part of the DNA/protein complex (Fig. 3B, lanes 5 and 6). The specificity of binding was demonstrated by competition with a molar excess of unlabeled wild-type competitor probe and by loss of retention by using a labeled probe containing both the putative *Pax2* and *Hox* binding sites mutated (Fig. 3B, lanes 4 and 7).

To confirm the importance of this Hox11-Eya1-Pax2 regulatory binding site in vivo, we generated *LacZ* reporter constructs by using wild-type and mutant *Six2* upstream sequences to test expression within the renal mesenchyme of transgenic mice. A 980-bp *Six2* construct was used to drive *LacZ* expression, and this was compared to expression from a construct containing mutations of the putative *Pax2* and *Hox* binding sites. The transgenic constructs were injected into fertilized mouse embryos and assayed for *LacZ* expression at E11.5. The wild-type *Six2* reporter demonstrated *LacZ* expression in a pattern similar to that of endogenous *Six2* expression (44) in 5 of 12 independent transgenic lines. *LacZ* staining was promi-

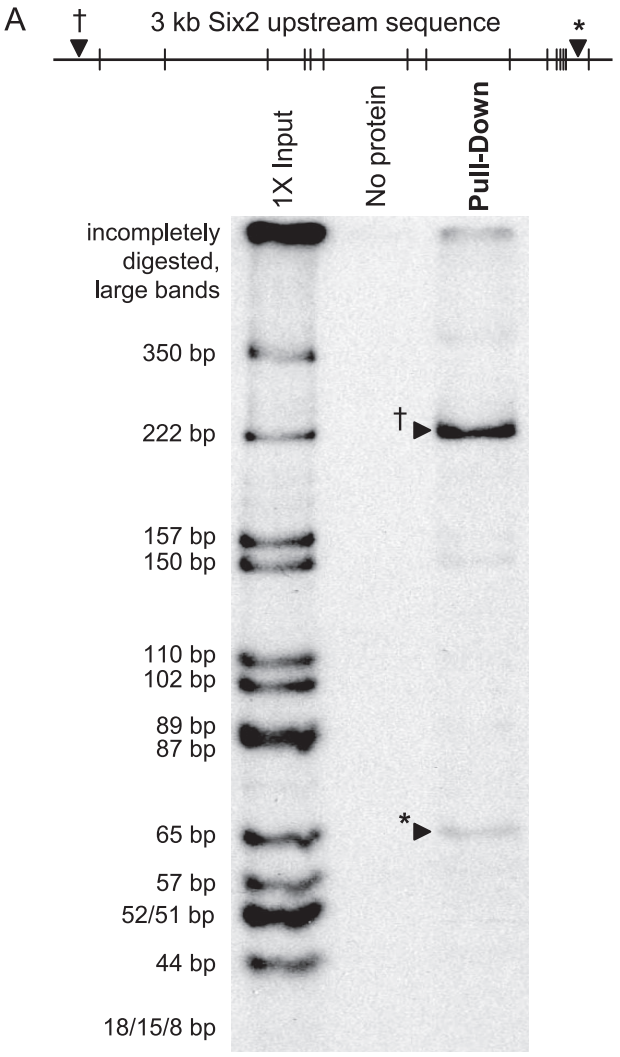

FIG. 2. Pax2 binds regions upstream of the Six2 protein coding sequence. (A) The Pax2-PD binds two regions of the *Six2* promoter in vitro. The black hatch marks indicate the HpaII sites in the 3.0-kb *Six2* upstream sequence. A 5′ 222-bp region (†) and a 3′ 65-bp region (*) are pulled down only when the Pax2-PD is present. (B) Sequence analysis of the 65-bp region at bp −450 identified a putative *Pax2* binding site and a putative *Hox* binding site based on sequence conservation to consensus sites.

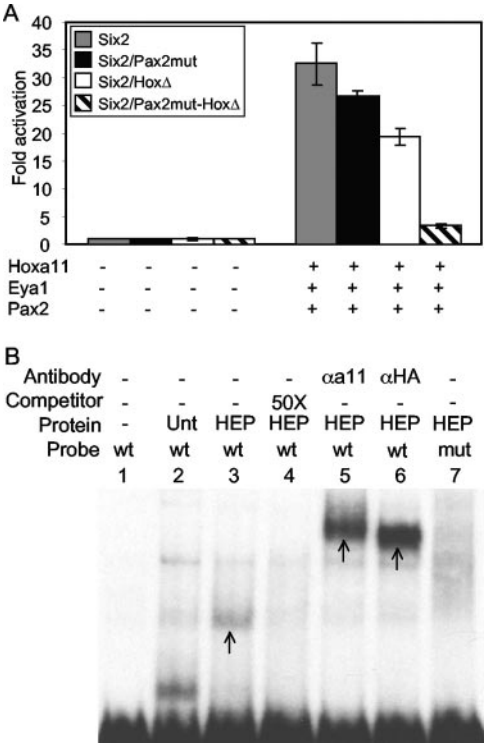

FIG. 3. The Hox11-Eya1-Pax2 complex binds at the bp −450 region site and is necessary for Six2 expression. (A) Luciferase activities from the 3.0-kb wild-type *Six2* expression construct (Six2) and constructs with the putative *Pax2* binding site mutated (Six2/Pax2mut), with the *Hox* binding site mutated (Six2/HoxΔ), or with both the putative *Pax2* and *Hox* sites mutated (Six2/Pax2mut-HoxΔ) were compared. All plates were cotransfected with or without Hoxa11, Pax2, and Eya1 protein expression vectors in MDCK cells. (B) An 89-bp probe (wt) containing the putative *Pax2* and *Hox* binding sites of the *Six2* promoter, incubated with nuclear extracts from HEK-293 cells transfected with Hoxa11, Eya1, and Pax2 (HEP), demonstrated retention on a nondenaturing acrylamide gel (arrow in lane 3). Probe retention was not seen in untransfected extracts (Unt; lane 2). This interaction was competed with excess (50×) unlabeled competitor (lane 4), and supershifts were observed using antibodies to Hoxa11 (αa11) or to an HA tag (αHA) on the Pax2 protein (arrows in lanes 5 and 6, respectively). The transfected extract does not show retention using a probe (mut) with the *Pax2* and *Hox* binding sites mutated (lane 7).

nent in the branchial arches, the otic region, and the developing urogenital mesenchyme (Fig. 4A). Of 26 independent transgenic embryos generated with the mutant *Six2-LacZ* reporter, 19 embryos demonstrated *LacZ* staining in some region of the developing embryo, but none of the 26 mutant embryos showed any staining in the nephrogenic mesenchyme (Fig. 4A). This confirms that the Hox11-Eya1-Pax2 binding site is necessary for *Six2* expression in the nephrogenic mesenchyme in vivo.

Both *Eya1* expression and *Pax2* expression are unaffected in the metanephric mesenchyme at preinduction stages in *Hox11* triple mutants (70). Further, *Eya1* expression is unaffected in *Pax2* mutant mesenchymes, and *Pax2* expression is initially unaffected in *Eya1* mutant mesenchymes (71, 73). We examined the expression of *Hoxd11* in the posterior nephrogenic mesenchymes of *Eya1* and *Pax2* mutant mice, and no changes in expression were seen (Fig. 4B to E). Therefore, while the loss of these genes individually leads to the loss of *Gdnf* expression and ureteric bud induction, these genes do not affect the expression of one another at early metanephric stages,

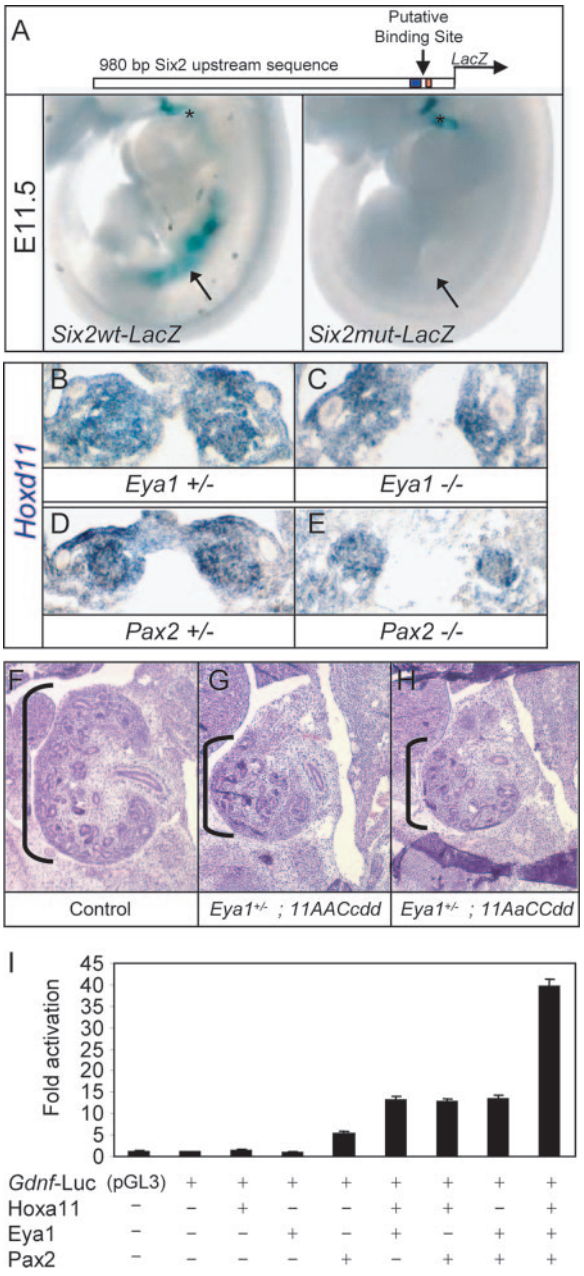

FIG. 4. The Hox11-Eya1-Pax2 binding site is critical for kidney expression in vivo, and the Hox-Eya-Pax network synergistically activates Gdnf expression. (A) *Six2-LacZ* transient transgenic mice. The top panel shows a schematic of the 980-bp *Six2-LacZ* reporters (*Hox* site in red and *Pax2* site in blue). (Lower left panel) E11.5 transgenic embryos carrying the wild-type *Six2-LacZ* constructs exhibit staining in the nephrogenic mesenchyme (arrow) and the branchial arches (asterisk). (Lower right panel) Transgenic mice carrying a construct with the *Pax2* and *Hox* sites mutated retain staining in the branchial arches (asterisk; 19 of 26 embryos) but have no nephrogenic staining (arrow; 26 of 26 embryos). (B through E) No differences in the expression of *Hoxd11* in the posterior intermediate mesoderm are seen in *Eya1* heterozygous (B) or homozygous (C) embryos at E10.5 or in the metanephric mesenchyme of *Pax2* heterozygous (D) or homozygous (E) embryos at E10.5. (F through H) Frontal hematoxylin-eosin-stained histological sections from an E14.5 control embryo (F) and embryos with three mutant *Hox11* alleles plus one mutant allele of *Eya1* (G and H). The brackets in panels F through H indicate relative kidney sizes. (I) *Gdnf* upstream sequence-driven luciferase activity in the presence of Hoxa11, Eya1, and/or Pax2 in MDCK cells.

consistent with their operating in parallel as transcriptional coregulators in this system.

If the Hox11-Eya1-Pax2 complex cooperatively contributes to the expression of early kidney mesenchyme-specific genes, then a reduction in gene dosage may provide genetic evidence for this interaction. *Eya1* heterozygotes or three-allele *Hox11* mutants have no histological renal phenotype at E14.5 (70, 71; data not shown). However, the addition of a single *Eya1* null allele to three mutant *Hox11* alleles results in hypoplastic kidneys at E14.5 (Fig. 4F to H). This phenotype is observed regardless of which three *Hox11* alleles are missing. Thus, reduced *Eya1* gene dosage uncovers a phenotype in the *Hox11* three-allele mutant kidneys similar to the one reported in mutants carrying four or more mutant *Hox11* alleles (11, 46, 70). These data provide compelling genetic evidence that Hox11 group proteins interact with Eya1.

Hox11 paralogous gene mutants and *Eya1* and *Pax2* mutant mice show similar kidney phenotypes with a failure to induce ureteric bud formation and loss of *Gdnf* (5, 70, 71). Thus, we examined *Gdnf* expression as a second potential candidate for regulation by the Hox11-Eya1-Pax2 complex. The *Gdnf* reporter construct was activated by Pax2 alone approximately fivefold, in agreement with previously published reports (5). However, coexpression of Hoxa11 (or Hoxc11 or Hoxd11 [data not shown]) with Eya1 and Pax2 increased activation of the *Gdnf* luciferase reporter more than 40-fold, whereas Hox11 or Eya1 alone had no effect on activation (Fig. 4I). These data demonstrate that the Hox11-Eya1-Pax2 complex can strongly activate multiple target genes in the early renal mesenchyme.

DISCUSSION

During the specification of the body plan, cells and tissues are organized along three axes: anterior-posterior, dorsal-ventral, and mediolateral. The intermediate mesoderm, from which the kidney derives, is first specified along the mediolateral axis and marks a region between the paraxial mesoderm and lateral-plate mesoderm shortly after gastrulation. One model proposes that Bmp signals from the lateral plate and as yet unidentified signals from the paraxial mesoderm provide positional cues to activate genes such as *Pax2*, *Osr1*, and *Lim1*, which mark the intermediate mesoderm (30, 31). The intermediate mesoderm also has anterior-posterior patterning that is clearly represented by the pro-, meso-, and metanephric kidneys. Thus, any position within the developing mesoderm can be specified by a unique combination of anterior-posterior factors and mediolateral factors. How these factors function cooperatively is unclear. Our results suggest a model whereby Hox11 paralogous proteins cooperate with Eya1 and Pax2 to activate *Six2* and *Gdnf*, two genes specific to the posterior intermediate mesoderm that generates the metanephric kidney (Fig. 5). Hox11, Eya1, and Pax2 proteins physically associate, bind to a metanephric-mesenchyme-specific enhancer region within the *Six2* promoter, and synergistically activate reporter gene expression. Thus, the direct interactions between anterior-posterior determinants and mediolateral determinants may be essential for determining the positional address of the metanephric mesenchyme.

Despite the fact that targeted deletions of *Hox* genes were among the first generated (8, 9, 35) and that mutations in *Hox*

FIG. 5. Diagram of a proposed mechanism of Hox11 molecular function. Taken together, this work supports a model wherein Hox11 proteins form a transcriptional complex with Pax2 and Eya1 and directly activate the expression of *Six2* and *Gdnf* during early mammalian metanephric development. Hox11P, a given Hox11 paralogous protein.

genes affect numerous developmental processes (reviewed in reference 47), few downstream target genes have been identified. Probably the most studied mammalian Hox target is *Hoxb1*, which is autoregulated by its own expression (13, 54). Working as a complex with Pbx and Meis/Prep, Hoxb1 has been shown to regulate several other anterior *Hox* genes (19, 20, 29, 36). Here, we demonstrate that *Six2* and *Gdnf* are novel molecular targets of the *Hox11* genes and identify a novel set of Hox regulatory partners, Eya1 and Pax2, for this activity.

The Pax-Eya-Six pathway is a conserved regulatory network in organogenesis (3). Initially found in *Drosophila*, *ey* (*Pax*) activates *eya* (*Eya*) and *so* (*Six*) expression during eye formation (22). *Eya* can also ectopically activate *so*/*Six*, and *so*/*Six* and *eya* both in turn regulate *ey*/*Pax* as well as their own expression (51). These genes are also expressed and play important roles in mammalian development. In addition to affecting kidney development, mutations in these genes affect development of other organ systems, such as ear, thymus, and thyroid formation, as well as muscle and eye formation (reviewed in references 3, 25, and 32). How this regulatory cassette contributes to the differentiation of so many unique structures is unclear but presumably relies on interactions with region-specific patterning factors.

More recently, it has been shown that Hox/HomC proteins can bind ey/Pax and affect developmental processes in *Drosophila* (1, 52). Our current work suggests that this interaction is conserved during mammalian kidney development as well. Further, it is possible that interaction between *Hox* genes and the *Pax-Eya-Six* pathway may be more generally conserved. For example, *Hoxa3* is necessary for thymus and thyroid formation, as are *Eya1* and *Six1* (37, 74, 76). *Pax1* and *Pax9* are also expressed in the thymus, and *Pax8* is expressed in the thyroid (42, 53, 69). Similarly, during otic development, *Hoxa2* is expressed in the second branchial arch, and *Hoxa2* mutant mice have middle ear defects, as do *Eya1*, *Six1*, and *Pax8* mutants (10, 21, 45, 56, 71). *Six2* is expressed in the first and second branchial arches and periotic mesenchyme, and a recent study has shown that *Six2* expression increases in these regions in *Hoxa2* mutants (33, 44). Because of the striking similarities in the phenotypes of these mutants, it is plausible that a Hox-Eya-Pax complex is also necessary for the development of other organ systems and that *Six* genes are general developmental targets of *Hox* genes and possibly this Hox-Eya-Pax complex.

In conclusion, our results demonstrate that a Hox11-Eya1-Pax2 regulatory network is necessary for *Six2* and *Gdnf* expression to promote mammalian kidney development. This study identifies new downstream targets of the *Hox11* paralogous genes as well as a novel regulatory complex in which these Hox proteins operate. It will be important to determine if this pathway is conserved in other organ systems during mammalian development.

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